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Patterns of Susceptibility of *Aspergillus* Isolates Recovered from Patients Enrolled in the Transplant-Associated Infection Surveillance Network[▽]

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We analyzed antifungal susceptibilities of 274 clinical *Aspergillus* isolates from transplant recipients with proven or probable invasive aspergillosis collected as part of the Transplant-Associated Infection Surveillance Network (TRANSNET) and examined the relationship between MIC and mortality at 6 or 12 weeks. Antifungal susceptibility testing was performed by the Clinical and Laboratory Standards Institute (CLSI) M38-A2 broth dilution method for amphotericin B (AMB), itraconazole (ITR), voriconazole (VOR), posaconazole (POS), and ravuconazole (RAV). The isolate collection included 181 *Aspergillus fumigatus*, 28 *Aspergillus niger*, 27 *Aspergillus flavus*, 22 *Aspergillus terreus*, seven *Aspergillus versicolor*, five *Aspergillus calidoustus*, and two *Aspergillus nidulans* isolates and two isolates identified as *Aspergillus* spp. Triazole susceptibilities were ≤4 μg/ml for most isolates (POS, 97.6%; ITR, 96.3%; VOR, 95.9%; RAV, 93.5%). The triazoles were not active against the five *A. calidoustus* isolates, for which MICs were ≥4 μg/ml. AMB inhibited 93.3% of isolates at an MIC of ≤1 μg/ml. The exception was *A. terreus*, for which 15 (68%) of 22 isolates had MICs of >1 μg/ml. One of 181 isolates of *A. fumigatus* showed resistance (MIC ≥ 4 μg/ml) to two of three azoles tested. Although there appeared to be a correlation of higher VOR MICs with increased mortality at 6 weeks, the relationship was not statistically significant ($R^2 = 0.61$; $P = 0.065$). Significant relationships of in vitro MIC to all-cause mortality at 6 and 12 weeks for VOR or AMB were not found.

Invasive aspergillosis (IA) is an important problem in immunocompromised patients, especially in persons who have received hematopoietic stem cell or solid organ transplantation. On the basis of recent treatment guidelines, voriconazole is recommended as the primary therapy for IA, with alternatives including lipid preparations of amphotericin B (AMB), caspofungin, micafungin, itraconazole (ITR), and posaconazole (POS) (28). In vitro resistance among *Aspergillus* species is uncommon and may be increasing (5, 9, 27, 30). Several studies report prevalence of triazole resistance of up to 4.2% among *Aspergillus* isolates, or as much as 2.1% among *Aspergillus fumigatus* isolates (13, 14, 23). Triazole cross-resistance has been reported in several studies (14, 18, 19). In contrast, other studies have described triazole resistance in <1% of isolates, even in the postvoriconazole era (8, 19). Because of the potential of increasing MICs to triazoles and widespread use of triazoles for IA treatment, surveillance of *Aspergillus* susceptibility, especially among isolates causing IA, is warranted.

Examining the influence of antifungal resistance on clinical outcomes has been challenging because of the difficulty in establishing large cohorts of IA patients with available isolates and because of the low frequency of resistant isolates. Moreover, a myriad of factors besides isolate susceptibility may contribute to patient outcomes. Recent reports describe the challenges of in vitro-in vivo correlations of *Aspergillus* spp. with current susceptibility testing methodologies (2, 17, 21). This challenge is especially daunting when considering the number of host- and transplant-related variables that impact outcomes of this infection (16, 25). While the impact of resistant *Aspergillus* isolates on outcomes has been demonstrated in animal models for triazoles, echinocandins, and AMB, a paucity of human data is available (2, 7, 12, 13, 17). Herein, we describe in vitro susceptibility patterns of *Aspergillus* isolates from transplant recipients with proven or probable IA col-

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lected as part of the Transplant-Associated Infection Surveillance Network (TRANSNET). In addition, the in vitro MIC in relationship to all-cause mortality is examined.

MATERIALS AND METHODS

Fungal isolates. TRANSNET, composed of 23 transplant centers throughout the United States, conducted prospective surveillance for invasive fungal infections from 2001 to 2006. *Aspergillus* isolates were recovered from transplant recipients with proven or probable IA, defined by modified European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria (3). Isolates were identified using morphological methods, when possible, by the participating center and sent to the Fungus Reference Unit at the Centers for Disease Control and Prevention (CDC), where identities were confirmed by morphology.

Nineteen (79%) of 24 TRANSNET centers contributed isolates. A total of 274 isolates from 255 unique cases were evaluated. Isolates were obtained from a variety of sources, but primarily from pulmonary specimens. The collection of isolates included 181 *A. fumigatus*, 28 *Aspergillus niger*, 27 *Aspergillus flavus*, 22 *Aspergillus terreus*, seven *Aspergillus versicolor*, seven *Aspergillus calidoustus*, and two *Aspergillus nidulans* isolates and two isolates identified only as *Aspergillus* species. Three *Aspergillus lentulus* and two *Neosartorya udagawae* isolates and one *Neosartorya fischeri* isolate identified by previously described molecular methods were also included (4). All isolates were grown on potato dextrose agar slants and stored at −70°C until testing.

Antifungal susceptibility testing. ITR (Janssen Research Foundation), voriconazole (VOR; Pfizer), POS (Schering-Plough), ravuconazole (RAV; Bristol-Myers Squibb), and AMB (Sigma Chemical Co., St. Louis, MO) were obtained as reagent-grade powders. The broth microdilution method was performed according to the CLSI M38-A2 standard (15). The final range of drug concentrations tested was 0.008 to 8 μg/ml for triazoles and 0.016 to 16 μg/ml for AMB. Following incubation, MIC endpoints were determined (visually) as the lowest drug concentration that prevented any discernible growth (optically clear), compared to that of the drug-free controls. For the purposes of this study we defined "resistance" to be MICs of ≥4 μg/ml for triazoles and MICs of >1 μg/ml for AMB (2, 11, 14, 18). Quality control was measured by inclusion of the following strains: *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, and *Aspergillus fumigatus* 204305. All quality control readings were within the recommended limits based on standard CLSI methodology (6).

Statistical methods. For analysis of the relationship between MIC and all-cause mortality at 6 and 12 weeks posttransplant, one isolate from each of the 255 patients was evaluated. When more than one isolate was available per patient, the isolate with the highest MIC was chosen. Chi-square or Fisher's exact methods were used to determine the association of MIC to all-cause mortality at 6 or 12 weeks for all isolates and by certain species, per drug received. The relationship of MIC to mortality was also analyzed with linear regression modeling. A second analysis, using the chi-square test, examined the relationship of an epidemiologic cutoff (MIC of >1 μg/ml for VOR) to mortality at 6 or 12 weeks for patients with *A. fumigatus* isolates who received voriconazole therapy (22). The epidemiologic cutoff was defined as the value obtained from an MIC distribution analysis after considering the mode of distribution and the reproducibility of the MICs. The mode ± 1 twofold dilution was obtained for VOR (22).

TABLE 1. In vitro susceptibilities of 274 *Aspergillus* isolates causing IA in transplant recipients

Species (no. of isolates)[a]	Antifungal agent	MIC (μg/ml)	
		Range	50%/90%
Aspergillus fumigatus[b] (181)	AMB	0.125–2	0.5/1
	ITR	0.125–4	0.25/0.5
	VOR	0.125–8	0.5/0.5
	POS	0.03–1	0.06/0.125
	RAV	0.25–1	0.5/1
Aspergillus niger (28)	AMB	0.125–0.25	0.125/0.25
	ITR	0.25–1	0.5/1
	VOR	0.5–1	1/1
	POS	0.06–0.5	0.25/0.25
Aspergillus flavus (27)	AMB	0.5–1	1/1
	ITR	0.06–0.25	0.125/0.25
	VOR	0.125–1	0.5/0.5
	POS	0.06–0.125	0.06/0.125
	RAV	0.25–0.5	0.5/0.5
Aspergillus terreus (22)	AMB	0.25–4	2/2
	ITR	0.03–0.25	0.125/0.25
	VOR	0.25–0.5	0.5/0.5
	POS	0.03–0.06	0.06/0.06
	RAV	0.5	0.5/0.5
Aspergillus versicolor (7)	AMB	0.125–1	0.5/1
	ITR	0.125–16	0.25/16
	VOR	0.25–2	1/2
	POS	0.06–16	0.25/16
	RAV	0.25–4	1/4
Aspergillus calidoustus (5)	AMB	0.5–1	1/1
	ITR	16	16/16
	VOR	4–8	4/8
	POS	16	16/16
	RAV	4	4/4
Other *Aspergillus* spp. (4)[c]	AMB	0.5–4	0.5/4
	ITR	0.06–0.25	0.125/0.25
	VOR	0.5–1	0.5/1
	POS	0.06–0.25	0.06/0.25

[a] POS was tested for 181 *A. fumigatus*, 27 *A. niger*, 13 *A. flavus*, 23 *A. terreus*, seven *A. versicolor*, six *A. ustus*, and 10 other *Aspergillus* species isolates. RAV was tested for 181 *A. fumigatus*, zero *A. niger*, 13 *A. flavus*, seven *A. terreus*, four *A. versicolor*, three *A. ustus*, and zero other *Aspergillus* species isolates.

[b] Includes three *A. lentulus*, two *Neosartorya udagawae*, and one *Neosartorya fischeri* isolates.

[c] Includes two *Aspergillus* species isolates and two *Aspergillus nidulans* isolates.

RESULTS AND DISCUSSION

Antifungal susceptibility testing results for four triazole drugs and AMB at 48 h are shown in Table 1. All triazoles demonstrated comparable degrees of antifungal activity with MICs of ≤4 μg/ml (POS, 97.6%; ITR, 96.3%; VOR, 95.9%; RAV, 93.5%). The triazoles were not active against five isolates of *A. calidoustus*, for which MICs were ≥4 μg/ml. In addition, one (14.3%) of seven isolates of *A. versicolor* was resistant to the triazoles tested, except VOR. Among the *A. fumigatus* isolates, only one (0.6%) of 181 isolates was triazole resistant, with MICs of ≥4 μg/ml for ITR and VOR. The MIC for this isolate was 0.25 μg/ml for POS, and RAV was not tested.

Low MICs to AMB were present for most isolates, with 93.3% of all isolates inhibited at an MIC of ≤1 μg/ml. The exception was *A. terreus*, for which 15 (68%) of 22 isolates tested had MICs of >1 μg/ml. One *A. nidulans* isolate had an MIC of 4 μg/ml, and two *N. udagawae* isolates had an MIC of 2 μg/ml.

Overall, resistant isolates were not uncommon in this collection, with resistance to triazoles in 10 (3.6%) or AMB in 18 (6.6%) of 274 isolates. When evaluating the relationship between MIC and overall mortality at 6 or 12 weeks in patients who received AMB or VOR, having either a triazole- or AMB-resistant isolate was not significantly associated with mortality by univariate analysis (Table 2). In addition, among *A. terreus* isolates, no difference in outcomes by MIC was noted (Table 2). With linear regression, as can be seen in Fig. 1, there was not a significant relationship between increasing VOR MIC

TABLE 2. Relationship between MICs and all-cause mortality in patients who received VOR and AMB therapy

MIC	Mortality (%) at[a]:	
	6 wk	12 wk
Isolates from patients who received AMB		
All *Aspergillus* isolates (n = 111)		
≤1 μg/ml	47/100 (47)	68/100 (68)
>1 μg/ml	3/11 (27) [0.20]	8/11 (72) [0.75]
A. terreus (n = 13)		
≤1 μg/ml	3/4 (75)	4/4 (100)
>1 μg/ml	2/9 (22) [0.07]	7/9 (78) [0.30]
Isolates from patients who received VOR		
All *Aspergillus* isolates (n = 115)		
<4 μg/ml	30/111 (27)	58/111 (53)
≥4 μg/ml	1/4 (25) [1.0]	2/4 (50) [0.93]
A. calidoustus (n = 3)		
<4 μg/ml	0	0
≥4 μg/ml	1/3 (33)	1/3 (33)

[a] P values are shown in brackets. P values were determined by chi-square or Fisher's exact method to assess association of mortality among patients with resistant versus nonresistant MICs. For the purposes of this study we defined "resistance" to be MICs of ≥4 μg/ml for triazoles and MICs of >1 μg/ml for AMB. Not all species are listed because of lack of resistant isolates. Some patients received VOR and AMB.

and mortality at 6 weeks (R^2 = 0.61; P = 0.065) or 12 weeks (R^2 = 0.18; P = 0.40) among patients who received VOR therapy. In contrast, an increasing AMB MIC was associated with decreased mortality at 6 weeks (R^2 = 0.70; P = 0.04); but not at 12 weeks (R^2 = 0.39; P = 0.18) (Fig. 2).

An additional analysis with use of an epidemiological cutoff for VOR was performed. The modal MIC for VOR among *A. fumigatus* isolates was 0.5 μg/ml, and the modal MIC ± 1 twofold dilution encompassed 96% of the strains for VOR (range, 0.25 to 1 μg/ml). Using a VOR cutoff of ≤1 μg/ml, the analysis did not show an association with all-cause mortality in patients with *A. fumigatus* infection (at 6 weeks, P = 0.28; at 12 weeks, P = 0.59).

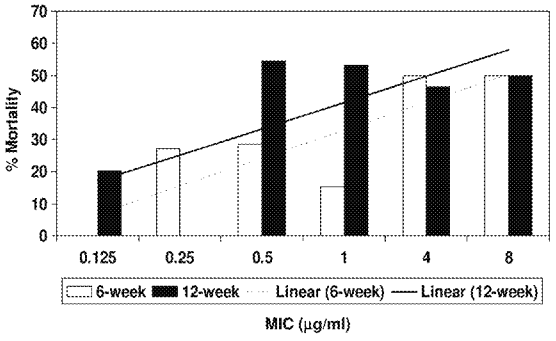

FIG. 1. All-cause mortality based on *Aspergillus* MIC among 115 patients who received VOR therapy. Regression lines for the association of MIC with mortality at 6 weeks (R^2 = 0.61; P = 0.065) and 12 weeks (R^2 = 0.18; P = 0.40) are shown.

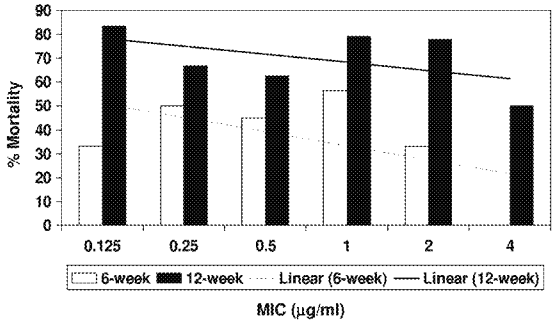

FIG. 2. All-cause mortality based on *Aspergillus* MIC among 111 patients who received AMB therapy. Regression lines for the association of MIC with mortality at 6 weeks (R^2 = 0.70; P = 0.04) and 12 weeks (R^2 = 0.39; P = 0.18) are shown.

This collection represents a large number of isolates from transplant patients with proven or probable IA collected from 19 U.S. institutions from 2001 to 2006. Overall, triazole agents showed good in vitro activity, with POS having the lowest MICs. Similar to recent reports, triazoles were active in over 95% of isolates (8, 19, 20). It is important to note that triazole resistance was uncommon among *A. fumigatus* isolates; only 1 of 181 isolates was resistant to two of four triazoles tested. This is in contrast to a recent report from medical centers in The Netherlands, in which ITR resistance occurred in 13 patients (12.8%) (23). It is important to note that our isolates came from transplant patients only, a very different population than that described by Snelders and colleagues (23). In addition, different susceptibility testing methods may be responsible for some variability. Finally, as Snelders and colleagues suggest, perhaps azole resistance among isolates from The Netherlands may be related to the use of azole fungicides. Our findings are similar to those seen in a recent U.S. survey, suggesting global geographic variation in susceptibility patterns (19). The impact of azole fungicides on resistance among U.S. isolates needs further exploration.

In the present study, all *A. calidoustus* isolates tested had MICs of >4 μg/ml for the triazoles. This newly described species, although uncommon, appears to be an emerging problem (1, 26). A recent report by Varga and colleagues (26) evaluated a large number of clinical and environmental *Aspergillus ustus* isolates, using phylogenetic analysis, and subsequently described *A. calidoustus* as a new species. All 27 *A. calidoustus* isolates were triazole resistant, with MICs of ≥8 μg/ml (26). Our findings reaffirm the elevated triazole MICs seen in *A. calidoustus* isolates. Of the five patients with *A. calidoustus*, only one (20%) died at the 12-week outcome endpoint, suggesting a potentially lower pathogenicity of this organism. Additional isolates and outcome data are required to confirm this.

Our study also confirmed earlier observations of the resistance of *A. terreus* to AMB (24, 29). *A. terreus* is reported in a wide range of immunocompromised patients. Correlations between in vitro and in vivo resistance and diminished therapeutic response have been reported for *A. terreus* infections with elevated MICs to AMB (10, 21). That our study did not demonstrate this correlation may be related to the relatively small

number ($n = 13$) of patients with *A. terreus* who received AMB and had outcome data available.

Our study evaluated the relationship between in vitro MICs and all-cause mortality at 6 and 12 weeks and did not confirm that "resistant" isolates were significantly associated with increased mortality, using several univariate analytic techniques. In addition, with use of an epidemiologic cutoff for VOR of ≤1 μg/ml among *A. fumigatus* isolates, no significant association was present (22). However, we were not able to confirm non-wild-type organisms with sequence analysis, and only 3 of 74 patients with *A. fumigatus* who received voriconazole therapy had isolates with MICs of >1 μg/ml.

Multiple factors contribute to patient outcomes, especially when considering all-cause mortality at 6 and 12 weeks, so the true impact of antifungal susceptibility is difficult to determine. We were not able to take into account length of therapy, dosages of antifungal agents, serum concentrations achieved, combination antifungal therapy, attributable mortality, and other factors that may impact outcomes in patients with IA. Most importantly, although this collection represents a large number of isolates, only 10% were either triazole or AMB resistant, resulting in a lack of power to determine differences. On the basis of these data, the authors do not recommend routine susceptibility testing of *Aspergillus* isolates, especially in areas of low frequency of resistance. However, in areas such as The Netherlands, routine testing may be more appropriate. If an isolate is discovered with an elevated MIC in a patient with aspergillosis, a thorough evaluation is needed to assess clinical response. Because of the myriad of factors that can impact patient outcomes, careful attention to antifungal therapy, drug levels, and patient factors is necessary, and a change in the antifungal regimen should be considered.

In summary, IA remains an important problem in transplant patients. Among transplant patients enrolled in this study, resistance to azoles was uncommon. Continued surveillance of *Aspergillus* susceptibility patterns and impact on patient outcomes is warranted.

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